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PRINCIPAL INVESTIGATOR: Jeffrey D. Rothstein MD, PhD

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21218

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Jeffrey D. Rothstein MD, PhD

E-Mail: jrothstein@jhmi.edu

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14. ABSTRACT

A growing body of evidence suggests that glial cells are critical for the support of normal neuronal function, and in particular astrocyte and oligodendroglial dysfunction may contribute to a number of neurodegenerative diseases, including ALS. One important function of glial cells is to transport nutrients from capillaries to neurons. Much of the nutritional support is in the form of glucose; however our lab and others have provided strong evidence that lactate support from *oligodendrocytes* via monocarboxylate transporters (MCTs) is a major contributor to neuronal metabolism and survival in vivo. Preliminary data from our laboratory indicates that genetic knockdown or pharmacological inhibition of glial specific monocarboxylate transporter 1 (MCT1) leads to loss of spinal cord motor neurons in vitro and in vivo. In addition, our lab and others recently showed in SOD1 mutant mice NG2+ cells, the progenitor cells for oligodendrocytes, upregulate NG2 and show enhanced proliferation in the ventral horn gray matter where motor neurons die. In addition, NG2+ cells formed large numbers of newly generated immature, non-myelinating oligodendroglia, but no astrocytes or neurons. Finally, when we specifically protected oligodendrocyte injury in the SOD1 animal model by deleting mutant SOD1 in NG2+ cells, we were able to delay disease onset by >126 days- many months longer than any other genetic ablation approach to date. We hypothesize that NG2 glial cells play a significant role in ALS pathogenesis. In particular, we are testing how human NG2 glial cells derived from ALS patient iPS cells will impact human motor neurons, also derived from human iPS cells.

15. SUBJECT TERMS
ALS, NG2 cell, myelin, therapy, oligodendroglia

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The role of human ALS iPSC-derived NG2 glia on human motor neuron survival: Strong implication of NG2 glia in ALS pathogenesis

Jeffrey D. Rothstein MD. PhD, Johns Hopkins University

Overall Summary

We successfully completed both aims of the DOD grant and were able to identify a major role for oligodendroglial and their precursors in ALS pathogenesis- first using rodent models and more recently using human ALS iPS cells.

Specific Aim 1: Differentiation and characterization of NG2 glia derived from ALS patient-specific iPS cells with A4V SOD1 mutation.

All experiments completed. Summary below. (including data previous progress reports)

1. Differentiation of ALS-iPS cells to NG2+ glial cells. Two lines of control iPS cells, and 2 lines of A4V-iPS cells and one A4V-carrier line were generated. The cells generated rosettes/neural tube structures which expressed NPC markers Pax6 and Sox1. There was no significant difference in the efficiency of neural tube formation (Figure 1). High efficiency of NPC generation was observed from both control and A4V iPS cell lines. No differences in NPC differentiation efficiency was seen between control and A4V lines. Immunocytochemistry did not show differences in NPC differentiation efficiency using two protocols. To generate NG2+ glial cells (OPCs), NPCs were further differentiated to pre-OPCs and OPCs, outlined in Figure 2, which showed reliable cell morphology and cell marker expression. Pre-OPCs were determined by their expression of Olig2 and NKX2.2 (Figure 1). The majority of colonies showed Olig2+, NKX2.2+, and Olig2+/NKX2.2+ cells, however the percentage of each marker expression varied among colonies.

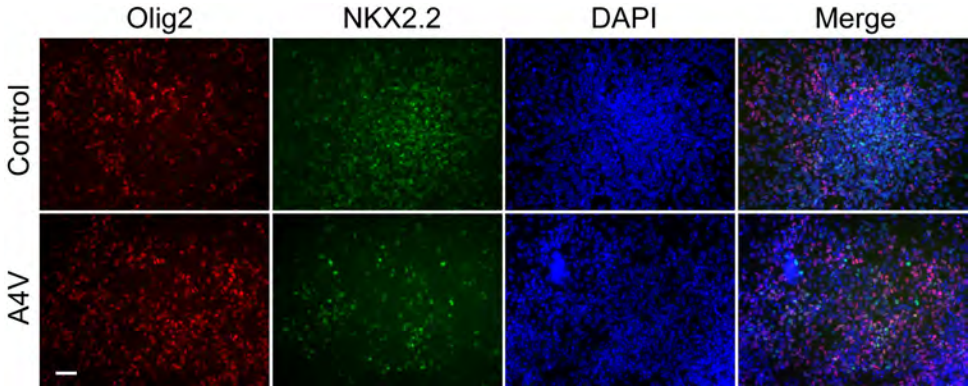
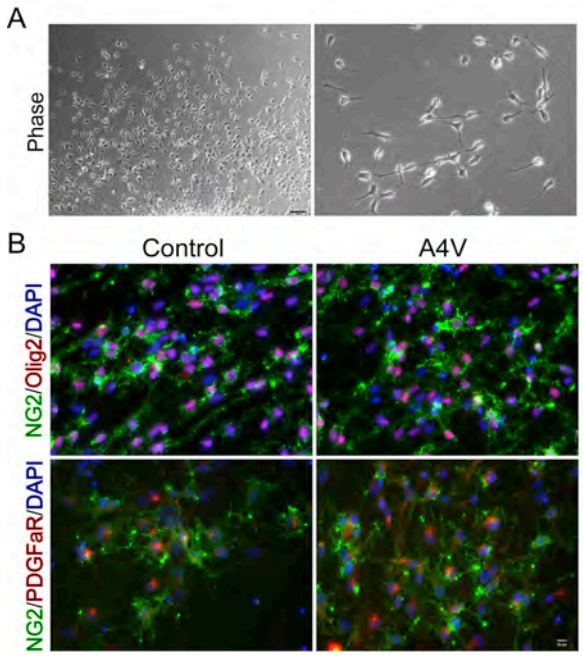


Figure 1. Pre-OPC generation by control and A4V-iPS cells. Spheres were attached around d35 and stained for marker expression 2-3 days later. Both control and A4V lines showed Olig2+/NKX2.2-, Olig2-/NKX2.2+, and Olig2+/NKX2.2+ cells. The positive cell number varies between colonies. Representative picutres were from 018 Control line and 013 A4V-line. Size bar, 50um.

To further differentiate pre-OPCs to OPCs, the spheres were cultured in the presence of PDGF, IGF and NT3. This step takes approximately 2 months. After about 120d differentiation, spheres were attached and cells migrated out from the spheres. Some had typical OPC morphology (figure 2A), bipolar and tripolar. More importantly, they expressed NG2, Olig2 and PDGFaR (figure 2B), all the OPC markers, indicating they are authentic OPCs.



2. Characterization of NG2+ glial cells.

To further characterize NG2+ glial cells, we first purified the cells using FACS. We took advantage of their cell membrane expression of PDGFaR, and labeled the cells with PE conjugated anti-CD140a (anti-PDGFaR), then sorted the cells (Figure 3A). The sorted cells showed NG2 and Olig2 expression (Figure 5B) after being cultured. After being treated with T3, some cells changed their bipolar and tripolar morphology to multiple process-bearing cells. These cells started to show O4 expression (Figure 3C and 3D). Slowly, the O4+ cells started to express the mature oligodendrocyte marker, MBP (Figure 3D). After at least about one month, there were some MBP+ oligodendrocytes with multiple processes and membranous structures (Figure 3E & 3F).

To determine whether $SOD1^{A4V}$ expression affect OPC gene expression, we did gene profiling analysis using Human Exon 1.0 ST Array (Affymetrix). As shown in Figure 4, principle component analysis showed that $SOD1^{A4V}$ expression probably interferes with gene expression based on PC1. In addition, A4V-patients and A4V-carriers may also have different gene expression profiles as suggested by PC2 analysis.

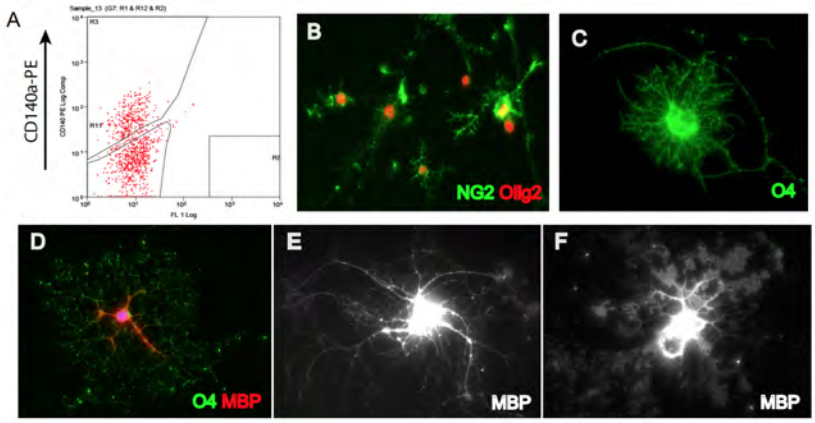


Figure 3. Differentiation of FACSed OPCs to mature oligodendrocytes. (A) OPCs were FACSed by their expression of PDGFaR (CD140a). (B) Sorted OPCs were cultured and expressed NG2 and Olig2. (C & D) O4 (green) expression by pre-oligodendrocytes differentiated from sorted OPCs. (D, E &F) mature MBP+ oligodendrocytes were differentiated from FACSed OPCs.

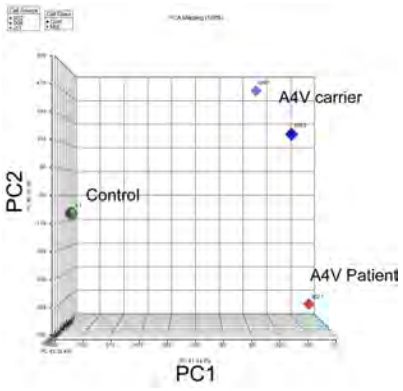


Figure 4. Principle component analysis on human OPCs. Control, A4V carrier and A4V patient specific OPCs were differentiated and purified by FACS based on their cell membrane expression of PDGFaR. Human exon array analysis was performed to compare their gene expression. Principle component analysis showed that A4V-carrier (blue, duplicate) and A4V patient (red) were separated from the healthy control (green) based on PC1.

<u>Specific Aim 2: Effects of NG2 cells with A4V SOD1 mutation on the survival of human motor neurons derived from patient-specific iPS cells</u>.

1. Differentiation and purification of motor neurons.

To differentiate control and A4V-11SOD1 iPSCs to motor neurons, the cells were differentiated to NPCs first and then to motor neuron. Motor neurons were defined by their expression of Hb9 or Islet1/2.

Given that during differentiation, neural progenitor or neuronal progenitors can give rise to new motor neurons. It is known that Hb9 expression is down regulated when motor neurons reach certain maturity. Therefore, it is necessary to use purified motor neurons in the coculture to limit the effects from newborn neurons or avoid the possible over estimation of neurotoxic effects due to the down regulation of Hb9. We observed that differentiated motor neurons at differentiation day 32 expressed Islet1/2 and Hb9. To successfully purify motor neurons, we took advantage of the expression of Hb9 by motor neurons during their development/genesis and transduce the cells with Lentivirus encoding Hb9-RFP as shown in published literature.

2. *Co-culture of human oligospheres and human mixed neurons.*

We explored whether human OPCs expressing SOD1^{A4V} are toxic to human neurons by co-culture of oligospheres and neurons. After about 3 weeks of coculture, more beaded axons were seen in the coculture with SOD1^{A4V} oligospheres compared to those with control oligospheres or neuron culture alone (figure 5). In the coculture of human oligospheres and human neurons, OPCs have been observed to migrate out from the spheres as seen in seeded oligosphere cultures in the absence of neurons. In the presence of neurons, oligodendrocytes were differentiated as indicated by their expression of MBP and they interacted with axons (figure 6). This reproducible result suggested that human oligodendrocyte can myelinate human neuron axons in the dish, although the efficiency was not high.

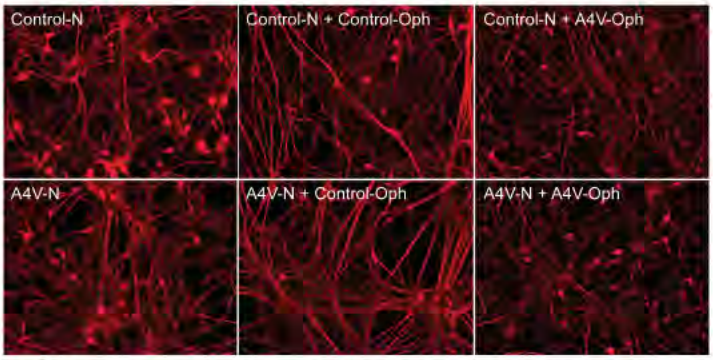


Figure 5. Axon degeneration in cocultures of human oligospheres and human neurons. Human Oligospheres were cocultured with human mixed neurons for about three weeks. Neurons were labeled by their expression of Tuj1 (red). Note beaded axons in the coculture with SOD1^{A4V} oligospheres.

3. *Co-culture of mouse OPCs overexpressing SOD1^{G93A} and human neurons.*

3.1 Overexpression of SOD1^{G93A} in mouse OPCs enhanced OL differentiation, but has no effects on OPC proliferation and cell death during oligodendrocyte differentiation, in the absence of neurons.

We evaluated mouse NG2 glia cells over-expressing G93A human SOD1 alone or in the presence of human neurons with and without A4V mutation. The reasons to use mouse G93A-NG2 cells are: 1) highly pure NG2 glia can be isolated from NG2-DsRed animals using FACS, and 2) the cells differentiate to mature oligodendrocytes (OLs) in a short time (5-6 days in the presence of T3).

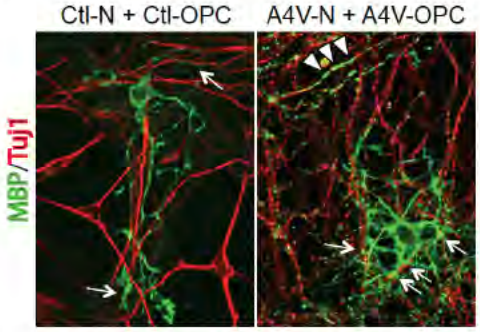


Figure 6. co-culture of human oligodendrocytes and human neurons. Human oligospheres were cocultured with human neurons. After 3 weeks of coculture, MBP (green) and Tuj1 (red) expression were determined by immunofluorescence staining. Arrows indicate the interaction of oligodendrocyte processes with axons. Arrowheads indicate possible myelination.

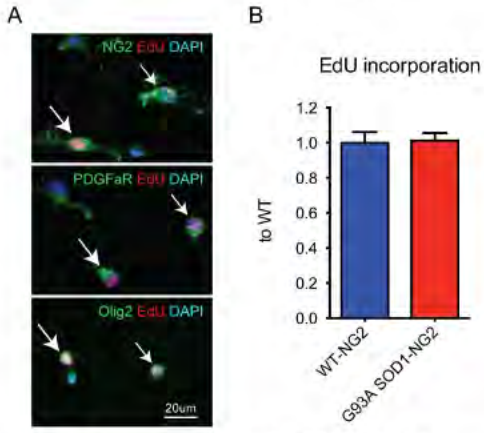


Figure 7. Overexpression of G93A SOD1 by mouse OPCs had no effects on cell proliferation. FACSed mouse NG2-DsRed cells were pulse traced with EdU for three hours and then Click-iT assay was used to determine EdU incorporation. (A) OPCs expressing NG2, PDGFaR or Olig2 (all in green) had EdU incorporation (red, arrows). Nuclei were stained with DAPI. (B) Quantification of EdU+ cells. EdU+ cells were by counted and divided by the total DAPI+ cells. Data was normalized to WT control. No differences in EdU incorporation have been seen between WT and G93A expressing OPCs (P>0.05, student t test).

To evaluate whether the overexpression of SOD1^{G93A} has effects on NG glia proliferation, differentiation and cell death during differentiation, EdU incorporation assay, efficiency of OL differentiation in the presence of T3, and TUNEL assay were performed respectively. No significant differences were observed in proliferation after a short pulse of EdU and cell death during OL differentiation (figure 7) between WT-NG2 and G93A-NG2 based on three independent experiments respectively. However, the presence of G93A-SOD1 slightly, but significantly enhanced OL differentiation (Figure 8). No differences in OL morphology have been seen between WT-OL and

G93A-OL (Figure 8). In addition, under unstressed condition, no ubiquitin+ aggregates were observed in G93A overexpressing cells.

Overexpression of G93A SOD1 enhances mouse oligodendrocyte differentiation, and possibly induce oligodendrocyte death in the presence of human neurons.

As overexpression of G93A SOD1 apparently enhanced NG2 glia differentiation to oligodendrocytes in the absence of neurons, we next asked whether the presence of neurons +/- mutant SOD1 affect mouse SOD1^{G93A} NG2 differentiation.

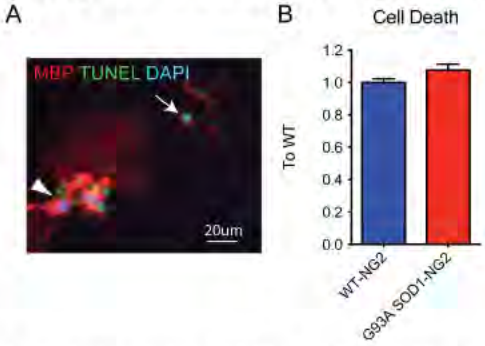


Figure 8. Overexpression of G93A SOD1 in mouse oligodendrocytes did not cause cell death during oligodendrocyte differentiation. (A) FACSed NG2-DsRed OPCs were differentiated to mature oligodendrocytes (MBP+) by T3 treatment. TUNEL assay was used to determine cell death after 5 day differentiation (dead cells are shown in green). (B) Quantification of dead cells. TUNEL+ cells were counted and divided by DAPI+ cells. Data was normalized to WT. (P>0.05, student t test)

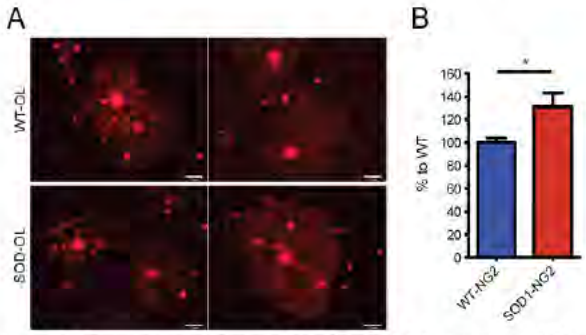
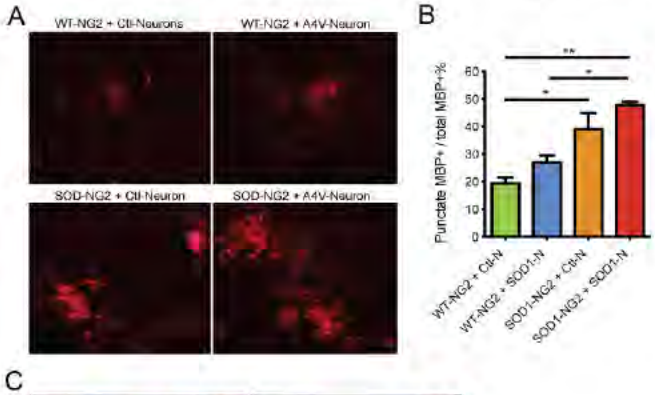
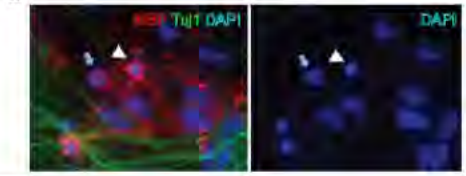


Figure 9. Overexpression of G93A SOD1 in mouse NG2 glia enhances oligodendrocyte differentiation. FACSed NG2-DsRed differentiated to oligodendrocytes in the presence of T3. (A) Mature OLs were determined by MBP expression (red). No obvious morphological differences in MBP+ cells have been noticed between the two groups. (B) Cell counting showed more MBP+ OLs were generated by G93A SOD1-NG2 cells (P<0.05, three independent experiments).

After 5-6 day differentiation, mature OLs were determined by their expression of MBP, and neurons were determined by Tuj1 expression. MBP+ mature OLs were generated in all co-cultures, while the staining intensity was much higher in the co-cultures with either SOD1-NG2 or A4V-neurons (Figure 9,10). All four groups had punctate MBP+ cells, however, in the co-cultures with G93A SOD1-NG2 glia the number of punctate MBP+ OLs was significant higher. Interestingly, some punctate MBP+ cells had condensed nuclear staining, indicating they are dying/dead cells (Figure 10). These data strongly suggest that neurons with mutant SOD1 enhance SOD1-NG2 glia differentiation to OLs, and these cells probably die after they differentiated.





3.2 *Overexpression of G93A SOD1 affects Wnt and Notch signaling pathways during mouse oligodendrocyte differentiation in the presence of neurons.*

Notch and Wnt signaling pathways play a role during oligodendrocyte differentiation and overexpression of G93A SOD1 enhanced oligodendrocyte differentiation in the presence of neurons. For this reason, we determined whether these two pathways were interfered in differentiating oligodendrocytes expressing G93A SOD1. Mouse specific Notch and Wnt PCR array

analyses were performed using the cross species co-cultures. Our data suggests oligodendrocytes with overexpression of G93A SOD1 had different gene expression profiles compared to WT no matter if they were cocultured with control or A4V-neurons (figure 14). Thus overexpression of G93A SOD1 interferes with oligodendrocyte differentiation.

3.3 Does mouse oligodendrocyte overexpressing G93A induce human neuron death?

We did not see strong evidence that mutant SDO1 oligodendrolgial could directly induce motor neuron death (Figure 12).

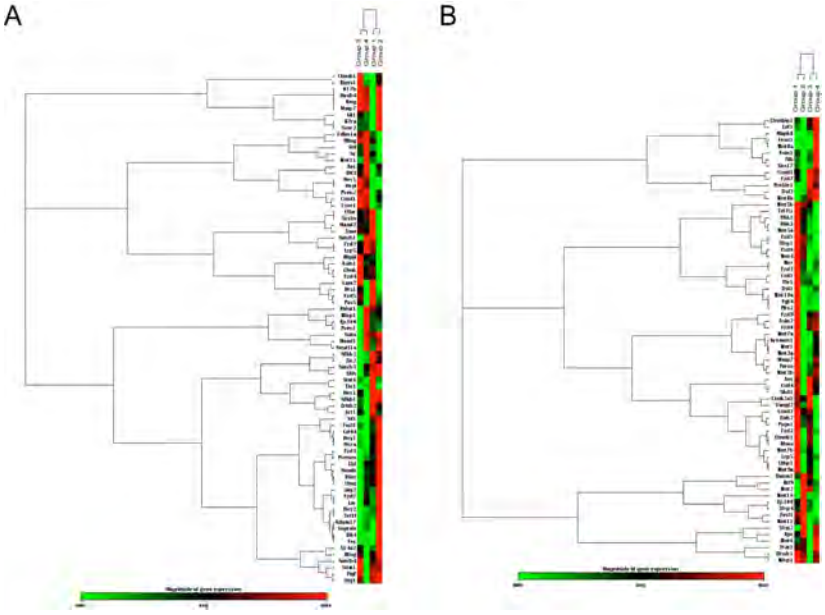


Figure 11. PCR array analyses on mouse Notch and Wnt signaling pathway. (A) Notch signaling pathway, (B) Wnt signaling pathway.

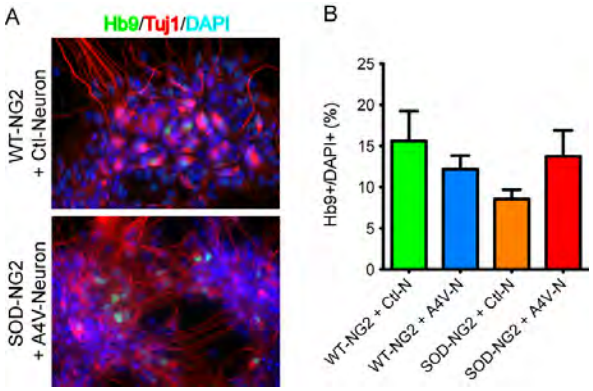


Figure 12. Motor neuron counts were not changed in the co-cultures of NG2 glia and neurons with and without mutant SOD1. (A) Mouse NG2 glia with and without G93A SOD1 were co-cultured with human control and A4V-neurons. Neurons and motor neurons were determined by the expression of Tuj1 (red) and Hb9 (green) after 5 days. Nuclei were stained with DAPI. (B) No significant differences in motor neuron numbers have been observed between each group (One-way ANOVA) in this experiment.

Progress towards on planned Milestones:

We achieved planned progress on all our orginal milestones for for the grant proposal:

- Differentiate NG2+ glial cells and oligodendrocytes from human iPS cells: ***COMPLETED***
- Characterize the differentiated cells at different stages of differentiation (immuno and RT-PCR): ***COMPLETED***
- Perform gene array analysis on differentiated NG2+ cells and oligodendrocytes: ***COMPLETED***

Key Research Accomplishments:

1. First successful differentiation of control and ALS mutant SOD1 iPS cells to NG2+ cells
2. First characterization of human ALS NG+/OPC iPS cells:
    a. Differentiation of OPC to mature oligodendroglial form ALS and control iPS cell
    b. First gene profile of ALS mutant SOD1 OPC iPS cells
3. First analysis of ALS NG2+ cell effet con the survival of cultured human motor neurons.
    a. First description of degeneration of motor neurons in the presence of mutant human OPC cells

Reportable Outcomes

1. Creation of ALS NG2 and oligodendroglial iPS cell

2. Discovery that human ALS oligodendroglial lead to degeneration of motor neurons

3. First model system to study human ALS oligodendroglia for both pathophysiology and drugs target screening

Conclusion:

Human ALS oligodendroglia appear to cause the axon degeneration of human ALS motor neurons , in vitro. Oligodendroglial appear to be actively causing ALS cell death.

References (Relevant publications toward Aim Progress):

Reported previously:
1. Lee, Y., B. M. Morrison, et al. (2012). "Oligodendroglia metabolically support axons and contribute to neurodegeneration." **Nature 487**(7408): 443-448

2. Kang SH, Li Y, Fukaya M, Lorenzini I, Cleveland DW, Ostrow LW, Rothstein JD, Bergles DE. Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. **Nat Neurosci**. 2013;16(5):571-9.

3. Morrison BM, Lee Y, Rothstein JD. Oligodendroglia: metabolic supporters of axons. **Trends Cell Biol**. 2013.